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Effect of three diets on the gametogenic development and fatty acid profile of *Paracentrotus lividus* (Lamarck, 1816) gonads

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Abstract

In this study, the effects of three diets were investigated to enhance *Paracentrotus lividus* production for commercial purposes. *P. lividus* were fed ad libitum for 80 days with: diet A—fresh *Codium tomentosum* Stackhouse, 1797; diet B—formulated using a jellified mix of macroalgae and vegetables, including *C. tomentosum* (20%), *Coralina* sp. Linnaeus, 1758 (17%), cabbage *Brassica oleracea* var. *capitata* Linnaeus, 1753 (30%), carrot *Daucus carota* Linnaeus, 1753 (30%) and agar (3%) as a gelling agent. Diet C consisted of maize *Zea mays Linnaeus*, 1753 (56%) and New Zealand spinach *Tetragonia tetragonoides* (Pallas, 1781) Kuntze, 1891 (44%). Their effects on the gonadal and somatic growths, gonadosomatic index (GI) and gametogenesis were evaluated, as well as on the total lipid content and fatty acid composition of sea urchin's gonads. Diet A provided high values of eicosapentaenoic acid (EPA). Gonads of sea urchins fed with diet A were found mostly in growth and maturation stages of gametogenesis and showed the lowest lipid content. Sea urchins fed with diet B presented their gonads in the reabsorption stage and had the highest values of omega-3 polyunsaturated fatty acids (PUFAs). Sea urchins fed with diet C were in the early stages of gametogenesis and had the highest values of lipid content, plus omega-6 PUFAs. Once as an ingredient in a balanced mix with vegetables, *C. tomentosum* can be a key factor to the development of new promising high-quality and low-cost feed for *P. lividus* roe enhancement.

KEYWORDS
echinoculture, EPA, gonadal enhancement, lipid profile, PUFA, roe, sea urchin

1 | INTRODUCTION

Sea urchin gonads (roe) are considered a delicacy, highly appreciated not only in Asia, but also in the North and South America, as well as in Europe, especially in the north region and in the Mediterranean (Bertocci, Dominguez, Freitas, & Sousa-Pinto, 2012; Bertocci et al., 2014; Carboni, 2013; Fernandez & Boudouresque, 2000; González-Irusta, Goñi de Cerio, & Canteras, 2010; McBride, 2005). An increase in market demand for the sea urchin *P. lividus* (Lamarck, 1816) has led to an overexploitation of this resource and an increase of illegal fishery (Boudouresque & Verlaquez, 2001; Pais, Serra, Meloni, Saba, & Ceccherelli, 2012; Prato, Fanelli, et al., 2018). Commercial sea urchin aquaculture has become mandatory to partially replace the decline in natural captures, plus to fulfil market needs with adult individuals, holding good-quality gonads, throughout all year long (Carboni, 2013).

Sea urchin aquaculture first began in Japan, in 1968, when natural fisheries were severely overexploited and, thus, unable to satisfy the expanding market (McBride, 2005). Intensive production practices in this country are performed in cage systems, in which sea urchins are fed with algae grown in captivity, until reaching the marketable size (Doumenge, 1990). Notwithstanding, the stocks of sea urchins have also declined in Europe, instigating countries to develop new aquaculture techniques. Despite this, the rearing methods for sea urchins' aquaculture are not well-established in the European countries, in contrast to other echinoid species worldwide. In an attempt to get ahead in this field, the number of studies on aquaculture and nutrition of sea urchins has grown exponentially in the last decade (Carboni, 2013; Carboni, Hughes, Atack, Tocher, & Migaud, 2013; Fabbrocini, Volpe, Coccia, D'Adamo, & Paolucci, 2015; Fabbrocini et al., 2012; James & Siikavuopio, 2015; Kelly & Chamberlain, 2010; Parisi et al., 2012; Prato, Chiantore, et al., 2018; Prato, Fanelli, et al., 2018; Sartori & Gaion, 2016; Sartori, Pellegrini, Macchia, & Gaion, 2016; Silva, 2012; Vizzini, Miccichè, Vaccaro, & Mazzola, 2015; Vizzini, Visconti, Vaccaro, & Mazzola, 2018; Zupo, Glaviano, & Paolucci, 2019).

In the European coastlines, *P. lividus* (Lamarck, 1816) is the most appreciated species of sea urchin. This invertebrate is valued by its high-nutritional content and its reddish-orange gonads, which owe their colour to the considerable presence of carotenoids (Sartori et al., 2016). Both male and female gonads are consumed, usually uncooked. They are also marketed salted, pickled or as a *pâté* (McBride, 2005).

Nowadays, aquaculture of sea urchins has emerged in two pathways: (a) production of juveniles for reseeding; and (b) gonadal growth and quality improvement in wild adult sea urchins (James & Siikavuopio, 2011; Pearce, Daggett, & Robinson, 2004; Robinson & Colborne, 1997; Spirlet, Grosjean, & Jangoux, 2000; Shpigel, McBride, Marciano, Ron, & Ben-Amotz, 2005). In their natural environment, sea urchins *P. lividus* predominantly fed on macroalgae, frequently *Laminaria digitata* (Hudson) Lamouroux, 1813, *Palmaria palmata* (Linnaeus) Weber & Mohr, 1805, *Ulva lactuca* Linnaeus, 1753 and *Porphyra umbilicalis* Kützing, 1843 (Boudouresque & Verlaquez, 2001; Cook & Kelly, 2007a; Ebert, 1982). Some authors described good results with mixes of these macroalgae (Doumenge, 1990; Frantzis & Grémare, 1992; Le Gall, 1987; McBride, 2005; Pearce et al., 2004; Schlosser, Lupatsch, Lawrence, Lawrence, & Shpigel, 2005). However, the use of macroalgae in aquaculture involves two main constraints: the fluctuating availability of good-quality seaweeds and its storage cost (Basuyaux & Blin, 1998). Therefore, the use of macroalgae is unlikely to be commercially viable in the form of a pure diet for mass production of sea urchins. In contrast, some land-based fresh vegetables, which may be constantly available, can be seen as a potential alternative. A waste recycling process of agricultural discards, combined with the use of high-value unprocessed biomass, could decrease the pressure in marine feed sources, such as fish meal or macroalgae (Vizzini et al., 2018). Many alternative feed sources have been studied to promote gonadal growth and to manipulate the gametogenic cycle for obtaining a desirable development stage. In particular, maize and spinach have been described as promising alternative diet sources (Basuyaux & Blin, 1998; da Rosa Repolho, Costa, de Jesus Luis, & de Matos Gago, 2011; Luis, Delgado, & Gago, 2005; Sartori & Gaion, 2016; Sartori et al., 2016; Sartori, Scuderi, Sansone, & Gaion, 2015; Silva, 2012). However, the formulation of a diet that can increase the size, enhance the nutritional value and the organoleptic characteristics of *P. lividus* gonads has not been fully achieved. Commercially acceptable gonads should be heavy and firm, with no release of gonadal fluids. Therefore, the sea urchins need to be sexually synchronized and their gonads must be in a specific stage of gametogenesis, in order to ensure a continuous production to supply the market demand for this product (Spirlet et al., 2000). It is currently thought that it is possible to control the reproductive cycle of sea urchins, using different water quality parameters and feed sources (Fabbrocini et al., 2015, 2012; Sartori & Gaion, 2016; Spirlet, Grosjean, & Jangoux, 1998, 2001; Walker, Harrington, Lesser, & Fagerberg, 2005; Walker, McGinn, Harrington, & Lesser, 1998). To create the appropriate diet for these purposes, it is necessary to understand the nutritional profile of sea urchins throughout their gametogenic development. In fact, it has been referred that dietary lipids have a key role in the fatty acid profile of roe (Carboni et al., 2013; Martinez-Pita, Garcia, & Pita, 2010; Siliani et al., 2016) and consequently, in their organoleptic attributes. Accordingly, three diets were tested, with macroalgae and vegetables, to compare the somatic growth of *P. lividus* sea urchins, but also the growth and nutritional quality of their gonads. The effects of these three diets were evaluated on the test diameter and individual wet weight of *P. lividus*, as well as on the gonadal wet weight, gonadosomatic index (GI), gametogenesis, total lipid content and fatty acid composition of the sea urchin gonads. The results attained in this work aimed to contribute for improving *P. lividus* rearing methods, for its sustainable aquaculture in a near future.

2 | MATERIAL AND METHODS

2.1 | Sea urchin collection

A total of 117 wild sea urchins *P. lividus* were collected haphazardly, by hand, in Praia do Abalo (Peniche, Portugal; 39°22'12.69"N; 009°23'7.07"W). Animals with a test diameter >3 cm were selected, to avoid the presence of juveniles (Ouréns, Fernández, & Freire, 2011). They were transported to the Aquaculture Laboratory of MARE—Marine and Environmental Sciences Centre of the Polytechnic Institute of Leiria. The sea urchins test diameter was measured with a Vernier caliper (±1 mm accuracy) (Lindner, Arnstorf, Germany) and their individual wet weight was assessed with an analytical balance AE ADAM PGL 3002 (Milton Keynes, United Kingdom; ±0.01 g accuracy).

2.2 | Starving period

Sea urchins do not have a natural synchronized gametogenic cycle. Therefore, it was necessary to establish a sexual synchronization

of the captured animals, in order to assess the influence of the experimental diets in the gametogenic development of their gonads. For this purpose, sea urchins were starved for 30 days, at a temperature of 16°C and salinity of 37 (Fabbrocini et al., 2012; Sartori & Gaion, 2016). Subsequently, sea urchins were randomly assigned in three recirculating aquaculture systems (RAS), each one composed by three tanks of 60 L, with aeration. Hence, there were 13 sea urchins in each 60 L tank, in a total of 39 individuals per RAS (n = 117 sea urchins; test diameter = 4.0 ± 0.4 cm; individual wet weight = 27.4 ± 7.7 g; mean ± *SD*). The water in each RAS was directed to a 96 L filtration system, with a biological filter (bio balls), a mechanical filter (glass wool), a water recirculation pump (Hailea Hx-6530, 1750 L/h, Guangdong, China), a protein skimmer (Bubblemagus SP1000, 300 L/h, Guangdong, China) and a refrigerator (FRIMAR® F500, Fernando Ribeiro Lda, Barcarena, Portugal).

Water quality parameters were assessed and recorded daily, as to guarantee the welfare of the sea urchins. Water temperature, salinity, pH and dissolved oxygen (mg/L) were measured with a multiparameter instrument YSI Professional Plus (YSI Incorporated, Yellow Springs, United States of America). Ammonia, nitrite and nitrate (ppm) were evaluated with rapid test kits API—Aquarium Pharmaceuticals (Mars Fishcare North America, Inc., Chalfont, Pennsylvania, United States of America). Frequently, one-third of the water volume was renewed in all the RAS, to avoid the deterioration of the water quality.

At the end of the starving period, three individuals per tank were randomly sampled (nine individuals per RAS). Their biometric characteristics were measured (test diameter, individual wet weight, gonads weight) and the respective GI was determined (Giese, 1959), to evaluate the effect of the three diets. In addition, gonads were also removed and fixed in buffered formaldehyde at 4%, for histological analysis (Fabbrocini et al., 2012; James & Siikavuopio, 2011).

2.3 | Experimental design

Three distinct feeds were administered to *P. lividus*, for 80 days, to evaluate their effects on the development of the sea urchins' gonads. Each feed was given to a group of 10 sea urchins, in one of the 60 L tank of each RAS. That is, a total of 30 sea urchins for each feed (90 individuals in total; test diameter = 4.0 ± 0.4 cm; individual wet weight = 27.4 ± 7.7 g; mean ± *SD*).

Water quality parameters were assessed daily as in the starvation period. Seawater parameters varied within the following values: temperature = 20.4 ± 1.4°C; pH = 8.0 ± 0.2; salinity = 37.0 ± 0.6; dissolved oxygen = 5.9 ± 0.5 mg/L; nitrogen compounds were maintained undetectable during the trial.

At the end of the experiment, all individuals were measured for their test diameter and individually wet weighed. Their gonads were also removed and wet weighed. One gonad per individual was removed and fixed in buffered formaldehyde at 4% for posterior histological analyses (James & Siikavuopio, 2011). The remaining four gonads, from each sea urchin, were individually conserved at −80°C, for further biochemical analyses.

2.4 | Diets and feeding

Sea urchins were fed with three different diets: diet A was composed of fresh *Codium tomentosum*, caught from the sea urchins sampling site; diet B was a jellified mix of macroalgae and vegetables; diet C consisted of maize *Zea mays* (56%) and New Zealand spinach *Tetragonia tetragonoides* (44%).

Codium tomentosum was chosen as a single ingredient diet (diet A), because it was one of the most abundant macroalgae present on the rock pools in Peniche. This macroalga was collected once in a week, in order to guarantee that it was given fresh to sea urchins.

Diet B was composed mainly by vegetables (60%), in order to understand if they were a good substitute of macroalgae in formulated feeds. It included the macroalga *C. tomentosum* (20%) and *Coralina* sp. (17%), the vegetables cabbage *Brassica oleracea* var. *capitata* (30%) and carrot *Daucus carota* (30%), plus agar (3%) as a gelling agent. All ingredients were put together in a blender and then a mixture of water and agar was added, according to the recommendations of Fabbrocini et al. (2012). In particular, the choice to use carrot was due to its amount of β-carotene (4,600 to 10,300 μg^{-100} g; Heinonen, 1990). Before the feeding experiment, diet B was tested for its stability in seawater, by maintaining it in aquaria for 3 days, without sea urchins. The feed did not lose biomass, disintegrate or loose consistency during this period.

Diet C was tested by other authors (Sartori & Gaion, 2016; Sartori et al., 2016,2015) and was well-accepted by sea urchins in captivity, allowing gonadal growth. Therefore, this diet was used in this study as comparing reference for diets A and B, as well as to juxtapose the results obtained with those pointed out by the previous works.

The sea urchins were daily fed ad libitum, during the trial (Basuyaux & Blin, 1998; Silva, 2012; Spirlet, Grosjean, & Jangoux, 2001). The food provided was weighed before being added into the tanks, as well as the food remaining in the bottom after 24 hr, to calculate the sea urchins' individual daily consumption (IDC) (Vizzini et al., 2015).

2.5 | Histology

After being fixated and preserved in buffered formaldehyde at 4%, the sea urchins' gonads were dehydrated and embedded in paraffin. Sections of 5 μm were obtained and stained with haematoxylin and eosin. It was possible to determine and characterize the different stages of gametogenesis, sexual ratio and oocyte diameter (5 measurements of 20 oocytes per female), by observing the permanent stained preparations, with an optical compound microscope Leica DM 2000 LED (Leica Microsystems GmbH, Wetzlar, Germany), equipped with a digital camera Leica MC 170 HD (Leica Microsystems GmbH, Wetzlar, Germany). The images were processed and analysed with the software Leica Application Suite 4.4.0 (Leica Microsystems GmbH, Wetzlar, Germany).

The characterization of the several stages of *P. lividus* gametogenic cycle was based on Byrne (1990).

2.6 | Biochemical analysis

Three replicates of each diet and the gonads of six random sea urchins, fed with each diet, were analysed for the total lipid content and fatty acid profile. The total lipid content was determined according to the methods of Bligh and Dyer (1959) and of De Coen and Janssen (1997). The fatty acid methyl esters (FAME) were prepared following the procedure of Lepage and Roy (1986) and analysed by gas chromatography (Thermo Electron Finnigan TRACE™ GC ultra-spectrometer; Thermo Fisher Scientific Inc., Milan, Italy), as described by Santos et al. (2016). FAME chromatographic peaks were identified by comparison of their retention times with those of standards Supelco® 37 (Supelco, Bellefonte, United States of America) and PUFA No.3 from Menhaden fish oil (Sigma-Aldrich, St. Louis, United States of America).

2.7 | Data analysis

The GI of *P. lividus* was determined as: GI (%) = gonad weight/wet weight × 100 (Giese, 1959).

The sea urchins' IDC was obtained as the difference between the wet weight of food provided (WFP) and the weight of the food retrieved (WFR) after 24 hr (Vizzini et al., 2015). The difference was then divided by the number of individuals (n) in each rearing tank, according to the formula: IDC (g ind^{-1} $days^{-1}$) = (WFP—WFR)/n.

Statistical analyses were performed in the statistical software package SigmaStat 4.0 (Systat Software Inc., San Jose, United States of America). The significance level considered in the statistical tests was $\alpha = 0.05$. A Mann–Whitney test was performed to compare sea urchins' test diameter before and after the starvation period. The same was done for the individual wet weight.

When the assumptions of normal distribution and homogeneity were met, one-way ANOVAs were performed to determine the existence of statistically significant differences between the biometric results of *P. lividus* subjected to the three different diets (test diameter, individual wet weight, before and after the assay), as well as of their IDCs and the development of their gonads (GI, gonadal weight, oocyte diameter), plus the biochemical analyses of total lipid and fatty acids contents of the three diets and in the sea urchins' gonads. Otherwise, when the tests of normality and homogeneity of the data did not meet the requirements, the non-parametric test Kruskal–Wallis on ranks was performed instead. When significant differences were found, a multiple-comparison *post-hoc* test was applied to search for the experimental groups that differed from the others. In this case, the Tukey HSD test (Honestly Significant Difference) or the Dunn test (when the normality of the data was not met and the sample size was unequal (Zar, 2010) was used.

3 | RESULTS

3.1 | Biometric data from the starvation period

No mortality was recorded during the starvation period.

There was a statistically significant difference between the initial and final test diameter (4.08 ± 0.46 cm and 3.95 ± 0.42 cm respectively; Mann–Whitney test: $U = 5{,}755$, $p = 0.035$). On the other hand, there was no statistically significant difference between the beginning and the end of the starvation period, regarding the mean individual wet weight (27.93 ± 8.25 g and 27.05 ± 7.70 g respectively; Mann–Whitney test: $U = 6{,}309$, $p = 0.301$). At the end of the starvation period, individuals presented a gonadal weight of 1.5 ± 1 g and a GI of 5.9 ± 3.5%.

3.2 | Biometric data from the feeding trial

In the beginning of the trial, there were no statistically significant differences between the test diameters of the sea urchins that would be submitted to the three diets (Table 1).

During the feeding trial, there was no mortality.

At the end of the trial, the test diameter did not show statistically significant differences between sea urchins fed with the different diets, as well as the individual wet weight (Table 1). Regarding the gonadal weight, sea urchins fed with diet C presented gonads

TABLE 1 Test diameter, individual wet weight, gonadal weight and gonadosomatic index of individuals fed with three different diets (Diet A—*Codium tomentosum*; Diet B—Jellified mix of macroalgae and vegetable; Diet C—Maize and spinach).

	Diet A	Diet B	Diet C	Statistical test	*p*-value
Beginning of the trial					
Test diameter (cm)	4.09 ± 0.34[a]	4.05 ± 0.41[a]	4.00 ± 0.40[a]	$H_{(2)} = 1.747$	$p = 0.417$
Individual wet weight (g)	27.78 ± 6.06[a]	27.69 ± 8.72[a]	26.72 ± 8.17[a]	$F_{(2,87)} = 0.175$	$p = 0.840$
End of the trial					
Test diameter (cm)	4.06 ± 0.52[a]	3.94 ± 0.42[a]	3.92 ± 0.45[a]	$F_{(2,89)} = 0.827$	$p = 0.441$
Individual wet weight (g)	27.78 ± 7,21[a]	29.10 ± 8.72[a]	29.05 ± 7.81[a]	$F_{(2,89)} = 0.265$	$p = 0.768$
Gonadal weight (g)	1.13 ± 0.71[a]	1.14 ± 0.80[a]	2.84 ± 1.72[b]	$H_{(2)} = 31.248$	$p < 0.001$
Gonadosomatic index (%)	4.48 ± 3.26[a]	3.80 ± 2.37[a]	9.97 ± 6.37[b]	$H_{(2)} = 33.811$	$p < 0.001$

Note: Results are presented as mean ± *SD*. Values signalled by different letters indicate significant differences between diets ($p < 0.05$), for which *F* stands for the results of one-way ANOVA and *H* for the Kruskal–Wallis tests

significantly larger than those from the individuals fed with diets A or B (Table 1).

Statistically, there were significant differences between diets, regarding sea urchins' GI. The most evident difference was observed for the individuals fed with diet C, who presented a GI significantly higher than those fed with diet A or diet B.

3.3 | Individual daily consumption (IDC)

The mean IDC was statistically different between all the three diets (Figure 1; Kruskal–Wallis test: $H_{(2)}$ = 152,22; p < 0.001). Diet B was well-accepted by the sea urchins, being the most consumed diet during the experimental period (11.00 ± 3.34 g ind^{-1} days^{-1}) (Figure 1). Diet A, being the most similar to the natural diet of *P. lividus*, was the second most consumed (6.72 ± 2.29 g ind^{-1} days $^{-1}$). Diet C was the least consumed by the sea urchins (3.00 ± 1.59 g ind^{-1} days $^{-1}$).

3.4 | Histology

The histological analysis of the gonads revealed that all of the sea urchins were in the stage I of the gametogenesis, at the end of the starvation period.

At the end of the experiment, all gametogenic development stages were observed (Figures 2 and 3), with noticeable variations between diets (Figure 4). Individuals from diet A were in recovery (stage I, 23%), growth (stage II; 23%) and mature (stage IV; 23%) stage of the gametogenesis. In diet B, about 37% of sea urchins were in recovery stage (stage I). In diet C, the greatest percentage of the individuals were in the recovery (stage I; 33%) and premature (stage III; 25%) stages of the gametogenesis.

Regarding the female gonads, no statistically significant differences were found between the oocyte diameter of sea urchins fed with the three diets (Kruskal–Wallis test: $H_{(2)}$ = 2,435; p = 0.296), presenting the following results: 41.83 ± 21.85 μm in diet A, 50.20 ± 19.83 μm for diet B and 44.99 ± 21.21 μm for diet C.

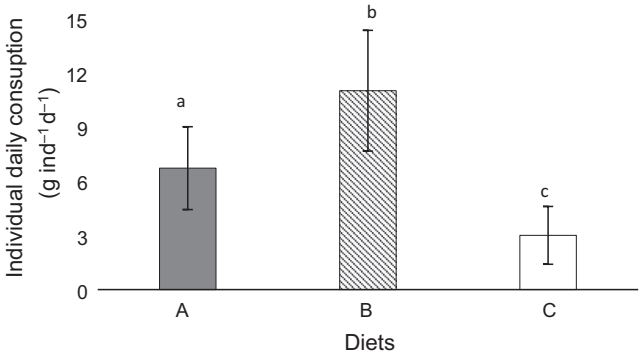

FIGURE 1 Individual daily consumption (IDC, mean ± *SD*) of sea urchins fed with three different diets. Diet A—*Codium tomentosum*; Diet B—Jellified mix of macroalgae and vegetables; Diet C—Maize and spinach. Different letters indicate statistically significant differences (p < 0.05)

3.5 | Biochemical analysis

3.5.1 | Total lipid content and fatty acid composition in *Paracentrotus lividus* diets

The total lipid content was statistically distinct amongst all the feeds given to *P. lividus* (Figure 5). Diet C presented the highest values (17.38 ± 2.1 mg/g), followed by diet A (9.26 ± 4.7 mg/g) and diet B presented the lowest values (1.65 ± 1.4 mg/g) (Kruskal–Wallis test: $H_{(2)}$ = 28.039; p < 0.001).

Diets A and B were composed by the same fatty acids (Table 2). Diet C was characterized by containing fewer saturated fatty acids (SFAs; 10.5 ± 2.8%) than diet A (22.1 ± 2.4%) and diet B (22.1 ± 7.1%), existing no significant differences on the amounts of total SFAs between these last two diets (Table 2). On the other hand, diet C had significantly higher amounts of monounsaturated fatty acids (MUFAs; 6.5 ± 1.2%) and polyunsaturated fatty acids (PUFAs; 11.3 ± 0.5%) relatively to diet A (1.3 ± 0.1% and 1.0 ± 0.1% respectively) and diet B (1.6 ± 0.6% and 1.5 ± 0.6% respectively), but there was no statistically difference between these two diets (Table 2). This pattern was also observed for both omega-3 and omega-6 PUFAs (Table 2).

Regarding SFAs, the stearic acid (C18:0) was the most abundant fatty acid. In diet A (12.68 ± 1.6%) and in diet B (12.79 ± 4.7%), the stearic acid was found to be significantly higher than in diet C (4.62 ± 1.8%) (Table 2). The three diets were rich in palmitic acid (C16: 0), representing 8.44 ± 0.8% in diet A, 8.2 ± 2.3% in diet B and 5.4 ± 1.1% in diet C, with no statistically significant differences between them (Table 2). The myristic (C14:0) and the arachidic (C20:0) acids represented less than 1% of the fatty acid composition of the different feeds (Table 2).

In what concerns MUFA, the oleic acid (C18: 1n9) was the most abundant in all diets (Table 2). Diet C showed to be significantly richer in oleic acid (6.25 ± 1.2%), when compared to diet A (1.3 ± 0.1%) and diet B (1.6 ± 0.6%), without significant distinction between these last two diets (Table 2). The palmitoleic acid (C16:1n-7) varied from 0.04% in diet A to 0.2% in diet C, without significant differences between the different diets (Table 2). The eicosenoic acid (C20:1n-9) was not found in diets A and B, but was in trace amount in diet C (0.1 ± 0.01%) (Table 2). The cis-vaccenic (C18:1n-7) and erucic (C22:1n-9) acids were not detected in all the three diets.

The linoleic acid (C18: 2n6) was the most abundant omega-6 PUFA (Table 2). It was remarkably higher in diet C (8.41 ± 0.6%), when compared with diet A (0.3 ± 0.01%) and diet B (0.9 ± 0.6%), in which diets A and B were not significantly different (Table 2). The docosapentaenoic acid (C22:5n-6) was 1.0 ± 0.02% of the fatty acid composition of diet C, but it did not make part of the biochemical composition of diets A and B (Table 2). The eicosadienoic (C20:2n-6) and arachidonic (C20:4n-6) acids were not detected in the three diets (Table 2).

In relation to omega-3 PUFA, the α-linolenic acid (C18: 3n3) was significantly higher in diet C (2.05 ± 0.4%) than in diet A (0.3 ± 0.1%) and diet B (0.2 ± 0.08%) (Table 2). The octadecatetraenoic acid (C18:4n-3) varied from 0.2% in diet C to 0.4% in diet A, without significant

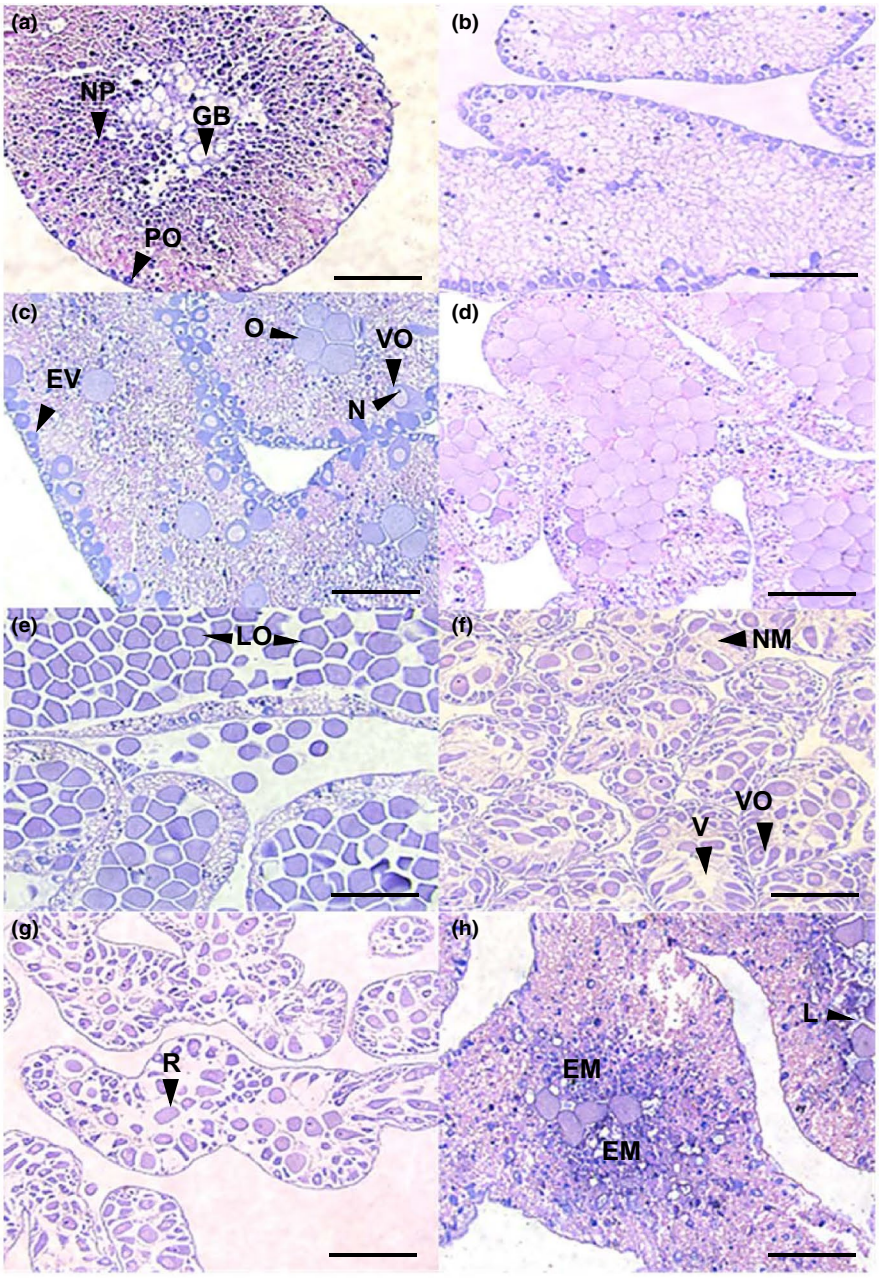

FIGURE 2 Development stages of the gametogenic cycle observed on *P. lividus* female gonads: a—Transition from spent stage (stage VI) to recovery stage (stage I): cross-section of the gonad, with visible globules (GB), derived from the lysis of non-released oocytes; noticeable nutritive phagocytes (NP) and pre-vitellogenic oocytes (PO) in the ascinal wall; b—Growing stage (stage II): Growth of oocytes and ovaries; c—Premature stage(stage III): premature ovary, with oocytes in all stages of development, namely early formed vitellogenic oocytes (EV) and vitellogenic oocytes (VO) that detached from the ascinal wall, as they turned into mature oocytes (O), in which the nucleus became more evident (N). d—Mature stage (stage IV): ovary replete with mature oocytes and a very small amount of nutritive phagocytes. e—Partly spawned stage (stage V): ovary with loose ovules (LO), lack of nutritive material and emergence of empty spaces, which indicated the beginning of spawning. f—Partial spawning of an ovary still in Stage III: oocytes at different stages of development, but mostly vitellogenic oocytes (VO) that eventually matured and moved to the lumen; nutritive phagocytes (NP); nutritious matter (NM); voids (V). g—Spent stage (stage VI): post-spawned ovary, presenting loss of the internal structure, which resulted in the presence of voids (V); oocytes that were not released, became dispersed inside the lumen, being later reabsorbed (R). h—Spent stage (stage VI): all the non-released vitellogenic oocytes, mature oocytes and ova will be later reabsorbed; nutritive phagocytes concentrated in the lumen, forming an eosinophilic meshwork (EM), in order to enclose and lyse the oocytes (L); the ovary began to reorganize. Histological slides stained with hematoxylin and eosin (all bars = 200 μm)

differences in the amounts of this fatty acid in the lipid composition of the three diets (Table 2). Diet A was distinct from diet B, due to the absence of the eicosapentaenoic acid (C20:5n3)—also known as EPA (diet A = 0%; diet B = 0,04 ± 0,03%). In diet C, EPA was slightly higher, even though no statistical differences supported this observation (Table 2). The eicosatrienoic acid (C20:3n-3) was only detected in diet

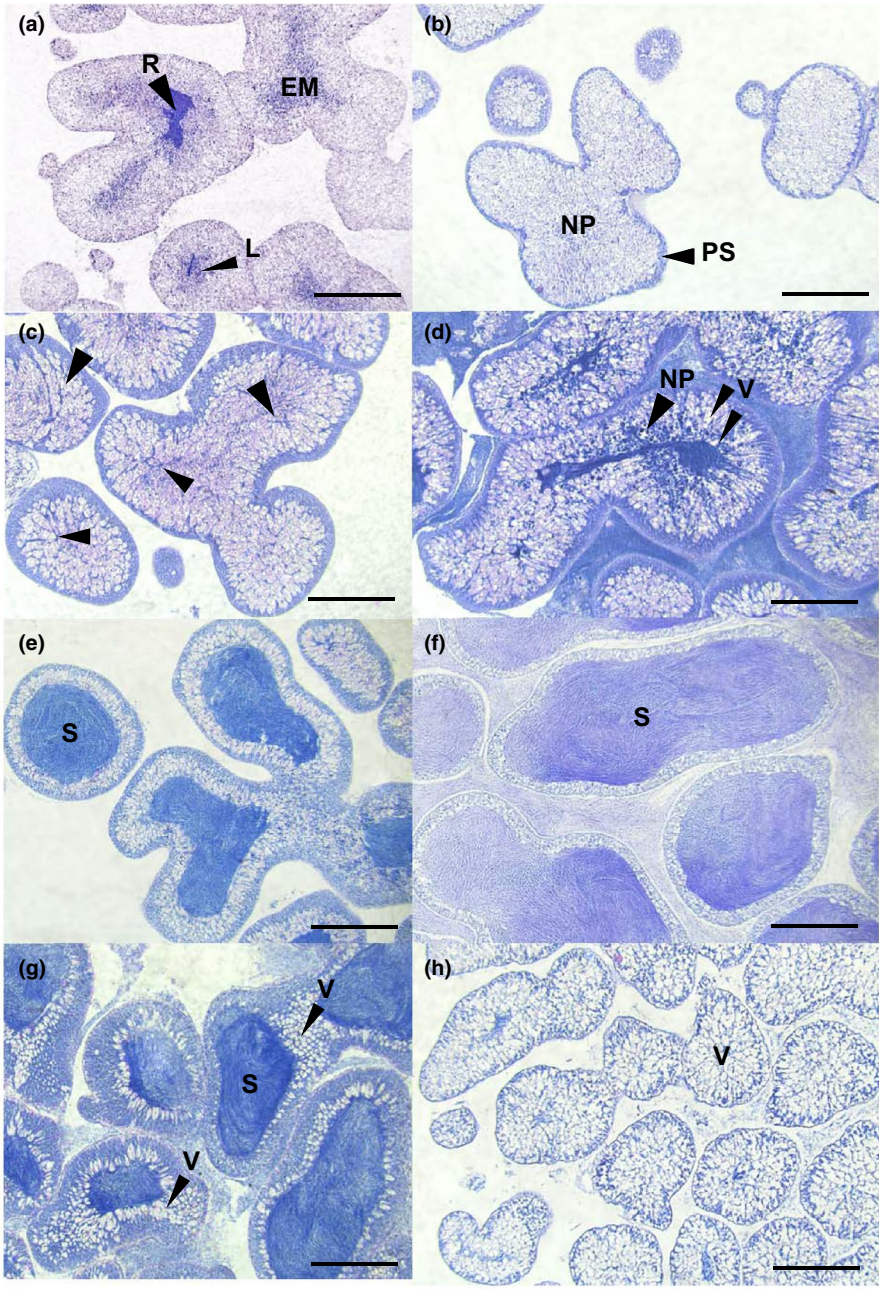

FIGURE 3 Development stages of the gametogenic cycle observed on *P. lividus* male gonads: a—Recovery stage (stage I): spawned testis with non-released spermatozoa that will be reabsorbed (R); nutritive phagocytes concentrated in the lumen (L), forming an eosinophilic mesh (EM) around the non-released spermatozoa. b—Recovery stage (stage I): cross-section of an early testis containing primary spermatocytes (PS) along the ascinal wall; a high amount of nutritive phagocytes (NP) began to appear. c—Growing stage (stage II): spermatocyte columns projected to the centre (arrows). d—Partial spawning of a testis in premature stage (stage III): nutritive phagocytes (NP) in the centre and empty spaces left by released spermatozoa (V). e—Premature stage (stage III): testis filled with mature spermatozoa (S) and largely devoid of nutrient tissue. f—Mature stage (stage IV): mature testis, filled with mature spermatozoa (S) ready to be released. g- Partly spawned stage (stage V): testis beginning to spawn, with voids (V) left by the released spermatozoa(S). h—Spent stage (stage VI): spent testis presenting voids (V) left by the released spermatozoa. Histological slides stained with haematoxylin and eosin (a, b, c, e, f, g and h's bars = 500 μm; D's bar = 200 μm)

C, in an amount that represented 0.1 ± 0.0% of its fatty acid composition (Table 2). No docosahexaenoic acid (C22:6n-3)—also known as DHA—was detected in the three diets (Table 2).

In what concerns the ω3/ω6 PUFA ratio, the three diets presented statistically significant differences among them. Diet A was the one presenting a higher ω3/ω6 ratio (2.0 ± 0.3), followed by diet B (0.8 ± 0.4) and by diet C (0.2 ± 0.1) (Table 2).

3.5.2 | Total lipid content and fatty acid composition in *Paracentrotus lividus* gonads

The gonads of individuals fed with diet C showed significantly higher values of lipid content (46.97 ± 25.99 mg/g) (Figure 6), followed by the gonads from sea urchins fed with the diet B (27.34 ± 7.37 mg/g) and diet A (21.39 ± 2.78 mg/g) (Kruskal–Wallis test: $H_{(2)} = 25.851$; $p < 0.0001$).

The sea urchins' gonads showed no statistically significant differences amongst the distinct diet groups, regarding the amounts of SFA, MUFA, omega-3 and total PUFA (Table 3). Nevertheless, SFAs were more abundant, varying from approximately 40% in sea urchins fed with diet C to 53% in sea urchins fed with diet A. PUFA, in the sea urchins' gonads, ranged from almost 28% with diet A to 37% with diet C. MUFAs were the least abundant, going from roughly 18% in diet A to 22% in diet C. However, the proportion of omega-6 PUFA was significantly higher in *P. lividus* fed with diet C (22.2 ± 3.7%) than those that were given diet A (4.7 ± 1.3%) or diet B (6.9 ± 4.7%), with no statistical differences found amongst this last two diets (Table 3).

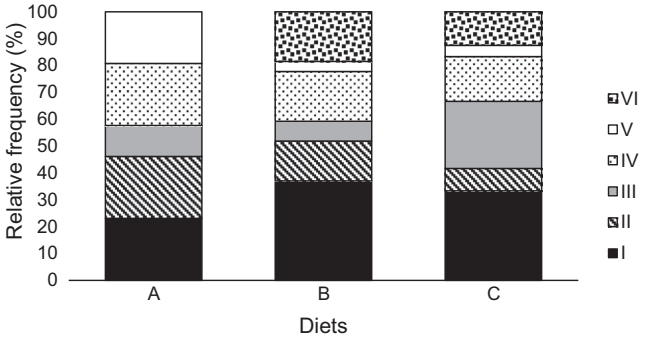

FIGURE 4 Relative frequency of the gametogenic stages identified in *P. lividus* fed with three different feeds. Diet A—*Codium tomentosum*; Diet B—Jellified mix of macroalgae and vegetable; Diet C—Maize and spinach. Stages: I—recovery stage; II—growth; III—premature; IV—mature; V—spawning; VI—spent

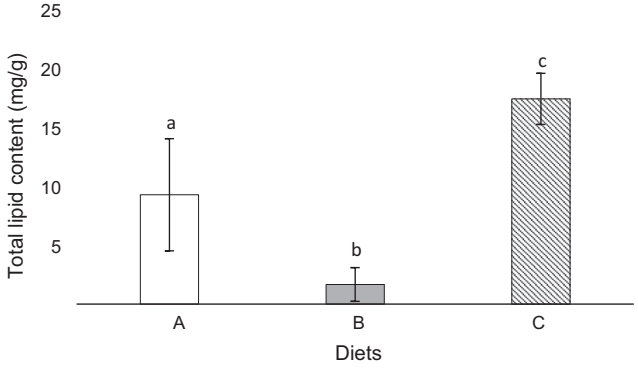

FIGURE 5 Total lipid content of the diets provided to *P. lividus* during the experimental trial (Diet A—*Codium tomentosum*; Diet B—Jellified mix of macroalgae and vegetables; Diet C—Maize and spinach). Different letters represent the statistically significant differences observed ($p < 0.05$)

In what concerns SFAs, the palmitic acid (C16:0) was the most abundant (≈ 22%–26%), followed by the stearic acid (C18:0 ≈ 11%–20%) and myristic (C14:0; ≈ 5%–7%) acids regardless of the type of diet (Table 3). Although the arachidic acid (C20:0) was the least abundant, its proportion was significantly distinct between gonads of sea urchins fed with diet A (1.2 ± 0.3%) and diet C (0.7 ± 0.3%). Notwithstanding the gonads of sea urchins fed with diet B did not differ from those that were given the previous two types of diets (Table 3).

The eicosenoic (C20:1n-9) and erucic (C22:1n-9) acids were generally the most abundant MUFA in the gonads of *P. lividus*, followed by the cis-vaccenic (C18:1n-7) and palmitoleic (C16:1n-7) acids. The oleic acid (C18: 1n9) was the only one that differed between diets. It was found in higher amounts in sea urchins from diet C (11.4 ± 3.1%) than those from diet A (3.2 ± 1.1%) or diet B (3.6 ± 1.01%), with no differences between these two last groups (Table 3).

In respect to the omega-6 PUFA, the linoleic acid (C18: 2n6) was the most abundant. The individuals fed with diet C presented the highest levels of this fatty acid (21.2 ± 3.7%), being statistically different from the others two diets. The arachidonic acid (C20:4n-6) was next. Its proportion differed between gonads from diets A (2.0 ± 0.6%) and C (0.7 ± 0.3%), but gonads from diet B (1.4 ± 0.5%) did not differ from those two. The docosapentaenoic acid (C22:5n-6) was not detected in the gonads of *P. lividus*, while the eicosadienoic acid (C20:2n-6) was the least abundant, varying from ≈ 0.6 to 1.3%, without significant differences between the different diet groups (Table 3).

The gonads of *P. lividus* sea urchins were rich in omega-3 PUFA, especially in those animals fed with diets A (23.2 ± 11.5%) and B (24.1 ± 10.5%) compared to diet C (14.9 ± 3.9%). Even though, no statistically significant differences were obtained between the three diet groups. The eicosatrienoic acid (C20:3n-3) was the most abundant in the sea urchins' gonads from all diets, representing from approximately 7% to 10% of the fatty acids. Also, the EPA (C20: 5n3; EPA) was very abundant, especially in diets A (9.8 ± 5.6%) and B (7.4 ± 6.5%), when compared to diet C (3.6 ± 2.5%). The α-linolenic (C18:3n-3) and octadecatetraenoic (C18:4n-3) acids ranged from nearly 2% to 5% of the fatty acid profile of *P. lividus* gonads. The docosahexaenoic acid (C22:6n-3; DHA) was detected in the sea urchins' gonads, in small amounts varying from closely 0.1% to 0.5% (Table 3).

In what concerns the ω3/ω6 PUFA ratio, gonads of *P. lividus* lividus fed with diet A or diet B presented higher values (5.2 ± 2.9 and 4.9 ± 4.2 respectively), which were significantly higher than in gonads of *P. lividus* fed with diet C (0.7 ± 0.3; Table 3).

4 | DISCUSSION

The echinoculture progress needs to conceive innovating feeds, in order to enhance and manage the sea urchins' growth and development, as well as improving the quality of the finishing product: the roe.

Some aspects of *P. lividus* nutrition have been studied by several authors, including the use of different macroalgae (Cook, Bell, Black, & Kelly, 2000; Cyrus, Bolton, Wet, & Macey, 2013; Doumenge, 1990; Frantzis & Grémare, 1992; Le Gall, 1987; McBride, 2005; Pearce et al., 2004; Prato, Chiantore, et al., 2018; Prato, Fanelli, et al., 2018; Schlosser et al., 2005), extruded and/or moist feeds (Cook & Kelly, 2007b; Fabbrocini et al., 2015, 2012; Lawrence, Olave, Otaiza, Lawrence, & Bustos, 1997; Prato, Chiantore, et al., 2018; Prato, Fanelli, et al., 2018; Sartori et al., 2016; Schlosser et al., 2005; Shpigel et al., 2005; Spirlet et al., 2001; Volpe et al., 2018; Zupo et al., 2019), along with fresh vegetables (Robinson & Colborne, 1997; Sartori & Gaion, 2016; Sartori et al., 2016; Silva, 2012; Vizzini et al., 2015, 2018). Namely, single macroalgal diet (Basuyaux & Blin, 1998; Gago, Luis, & Repolho, 2009; Luis et al., 2005; Silva, 2012), as well as maize and spinach, have often been used to feed sea urchins in captivity (Basuyaux & Blin, 1998; Sartori & Gaion, 2016; Sartori et al., 2016,2015; Silva, 2012). The present work compared the use of an agar-based diet containing macroalgae and vegetables against a single macroalgal diet (*C. tomentosum*) and a diet combination of maize and spinach. However, the use of macroalgae as a food source for sea urchins' nutrition is an expensive alternative with environmental

TABLE 2 Fatty acid composition (%) of the experimental diets (Diet A—*Codium tomentosum*; Diet B—Jellified mix of macroalgae and vegetables; Diet C—Maize and spinach) given to *P. lividus* during the experimental trial: saturated fatty acids (SFAs); monounsaturated fatty acids (MUFAs); omega-6 polyunsaturated fatty acids (ω6); omega-3 polyunsaturated fatty acids (ω3).

Fatty acids	*Diet A*	Diet B	Diet C	Statistical test	*p-value*
SFA					
Myristic (C14:0)	0.6 ± 0.1[a]	0.6 ± 0.2[a]	0.2 ± 0.1[b]	$F_{(2,6)} = 11.949$	0.008
Palmitic (C16:0)	8.4 ± 0.8	8.2 ± 2.3	5.4 ± 1.1	$F_{(2,6)} = 0.361$	0.058
Stearic (C18:0)	12.7 ± 1.6[a]	12.8 ± 4.8[a]	4.6 ± 1.8[b]	$F_{(2,6)} = 6{,}926$	0.028
Arachidic (C20:0)	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.04	$F_{(2,6)} = 0.522$	0.756
MUFA					
Palmitoleic (C16:1n-7)	0.04 ± 0	0.09 ± 0	0.2 ± 0.2	$F_{(2,6)} = 1.473$	0.349
Cis-vaccenic (C18:1n-7)	nd	nd	nd	–	–
Oleic (C18:1n-9)	1.3 ± 0.1[a]	1.6 ± 0.6[a]	6.2 ± 1.2[b]	$F_{(2,6)} = 37.648$	<0.001
Eicosenoic (C20:1n-9)	nd[a]	nd[a]	0.1 ± 0.01[b]	$F_{(2,6)} = 89.286$	<0,001
Erucic (C22:1n-9)	nd	nd	nd	–	–
ω6 - PUFA					
Linoleic (C18:2n-6)	0.3 ± 0.01[a]	0.9 ± 0.6[a]	8.4 ± 0.6[b]	$F_{(2,6)} = 251.569$	<0.001
Eicosadienoic (C20:2n-6)	nd	nd	nd	–	–
ARA—Arachidonic (C20:4n-6)	nd	nd	nd	–	–
DPA—Docosapentaenoic (C22:5n-6)	nd[a]	nd[a]	1 ± 0.02[a]	$F_{(2,6)} = 3.997$	0.079
ω3 - PUFA					
α-Linolenic (C18:3n-3)	0.3 ± 0.1[a]	0.2 ± 0.08[a]	2.1 ± 0.4[b]	$F_{(2,6)} = 63.170$	<0.001
Octadecatetraenoic (C18:4n-3)	0.4 ± 0	0.3 ± 0.2	0.2 ± 0	$F_{(2,6)} = 6.709$	0.707
Eicosatrienoic (C20:3n-3)	nd[a]	nd[a]	0,1 ± 0[a]	$F_{(2,6)} = 10.934$	<0.001
EPA—Eicosapentaenoic (C20:5n-3)	nd[a]	0.04 ± 0.03[a]	0.06 ± 0.04[a]	$F_{(2,6)} = 3.959$	0.080
DHA—Docosahexaenoic (C22:6n-3)	nd	nd	nd	–	–
Σ SFA	22.1 ± 2.4[a]	22.1 ± 7.1[a]	10.5 ± 2.8[b]	$F_{(2,6)} = 30.494$	0.012
Σ MUFA	1.3 ± 0.1[a]	1.6 ± 0.6[a]	6.5 ± 1.2[b]	$F_{(2,6)} = 21.392$	<0.001
Σ PUFA	1.0 ± 0.1[a]	1.5 ± 0.6[a]	11.3 ± 0.5[b]	$F_{(2,6)} = 31.721$	<0.001
Σ ω3	0.7 ± 0.1[a]	0.6 ± 0.2[a]	2.3 ± 0.5[b]	$F_{(2,6)} = 25.759$	0.001
Σ ω6	0.3 ± 0.01[a]	1.0 ± 0.7[a]	9.1 ± 0.1[b]	$F_{(2,6)} = 416.938$	<0.001
ω3/ω6	2.0 ± 0.3[a]	0.8 ± 0.4[b]	0.2 ± 0.1[b]	$F_{(2,6)} = 6.794$	<0.001

Note: Results are presented as mean ± *SD* (*n* = 3) and 'nd' stands for a fatty acid not detected in the diet composition. Values signalled by different letters indicate significant differences between diets ($p < 0.05$), for which *F* stands for the results of one-way ANOVA tests

issues. The main problem is the intensive macroalgae harvesting that may endanger the delicate dynamic equilibrium of coastal ecosystems. Therefore, the use of prepared diets, with low macroalgae content, can be a desirable improvement. The advantage of prepared feeds on gonadal growth is known for the sea urchins *Loxechinus albus* Molina, 1782, *Evechinus chloroticus* Valenciennes, 1846 and *Psammechinus miliaris* (P.L.S. Müller, 1771) (Barker, Keogh, Lawrence, & Lawrence, 1998; Cook, Kelly, & McKenzie, 1998; Lawrence et al., 1997). Fabbrocini et al. (2012) fed *P. lividus* with three agar-based diets containing different macroalgae and suggested that this type of feeds may represent a useful resource for the nutrition of sea urchins in rearing conditions.

In this study, the starvation period proved to be an essential procedure to synchronize the reproductive stage of *P. lividus* gonads, since the sea urchins were in stage I (stage in which there is reabsorption of the unreleased gametes). The results obtained met those of previous works, which suggested that starvation does not necessarily cause a decrease in the volume of the gonads, but does revert the gonads to the initial stages of gametogenesis (Guillou, Lumingas, & Michel, 2000). Indeed, sea urchins deprived of food may use the reserves contained in their nutritive phagocytes (Walker et al., 2005, 1998). Therefore, starvation at low temperatures can lead to a reduction in gonadal weight and an associated regression to the initial reproductive stages (Fabbrocini et al., 2012; Sartori & Gaion, 2016; Spirlet, Grosjean, & Jangoux, 1998). Once the organisms are sexually synchronized, their gametogenic development can be properly manipulated to achieve a desirable stage, when their gonads may be sold as good quality and palatable roe (Fabbrocini et al., 2015, 2012; Sartori & Gaion, 2016; Silva, 2012; Spirlet et al., 2001). From the commercial point of view, this may be a key procedure for

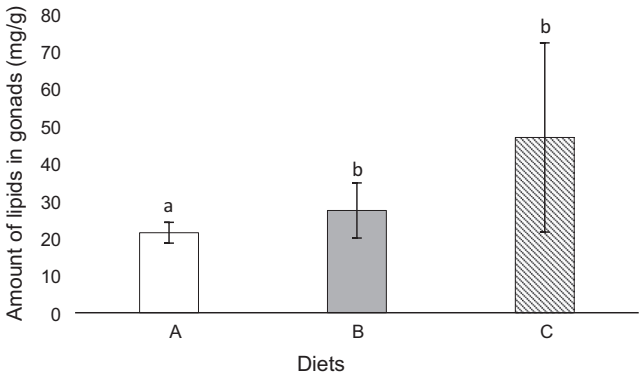

FIGURE 6 Total lipid content present in the gonads of *P. lividus* sea urchins, fed with three different diets (Diet A—*Codium tomentosum*; Diet B—Jellified mix of macroalgae and vegetables; Diet C—Maize and spinach), at the end of the experimental trial. Different letters represent the statistically significant differences observed ($p < 0.05$)

the aquaculture of sea urchins, accomplishing a continuous production in successive batches, in order to reply the market demand for these animals. Moreover, some authors defend that sea urchins may experience a decrease in test diameter when subjected to periods of starvation (Lawrence & Lane, 1982; Régis, 1979). Sea urchins' ossicles are constructed as a fenestrated stereom made of calcite, which varies in terms of porosity and construction (Ebert, 2007). Sea urchins' growth occurs by calcification around individual plates and by the development of new plates in the ambulacral and interambulacral rows at the aboral side of the test (Ebert, 2007). Although, reabsorption of calcite seems to be recurrent during starvation periods in echinoderms, there is some controversy regarding this subject. In the case of sea urchins, measuring the test diameter is a challenging task, specifically to accurately put the calipers' jaws between the spines. Therefore, measurement errors of few millimetres may occur, which can account for some bias results of test shrinkage, especially when this procedure is carried out by more than one operator (Ebert, 2007)—which was not the present case. In this study, *P. lividus* decreased in size after a 30-day period of starvation and did not recover on because to the fact that sea urchins may have allocated energy and nutrient resources for gonadal growth and gametogenic development, instead of expending it on somatic growth (Guillou et al., 2000). Other studies have also shown that feeding does not always promote somatic growth (Fernandez & Galtagirone, 1994). Guillou et al. (2000) reported that the sea urchin *Sphaerechinus granularis* (Lamarck, 1816), when in good nutritional conditions, did not increase in size during the mature stage, but allocated energy for gonadal growth and stored reserves in the body wall.

The use of maize has been the subject of several studies on sea urchins' nutrition (Basuyaux & Blin, 1998; Sartori & Gaion, 2016; Sartori et al., 2016,2015; Schlosser et al., 2005; Silva, 2012; Spirlet et al., 2001). Accordingly, the diet C used in this work was based on maize and spinach. Despite being the least consumed feed, it showed a better performance in increasing the weight of the gonads and boosting the GI. In order to have a good-quality feed, available all year round, obtained at low cost, causing low-environmental impact and that does not compete with food resources for human consumption, it is imperative to search for new sources and appropriate materials. Accordingly, Vizzini et al., (2015) used different kinds of vegetables discards (beet *Beta vulgaris* Linnaeus, 1753, cabbage *B. oleracea* and lettuce *Lactuca sativa* Linnaeus, 1753) and the sea lettuce macroalgae *Ulva lactuca* Linnaeus, 1753 as different feeds for *P. lividus*. The vegetable-based diets were able to maintain the initial values of the GI, while the *U. lactuca* diet caused a decrease in the same parameter. Vizzini et al., (2018) also used *L. sativa* for small-sized specimens (test diameter: 16.64 ± 0.93 mm, mean ± *SE*) and the sea urchins' test diameter and total weight increased by 35% and 56%, respectively, at the end of the trial.

Agar is widely used as a gelling and binding agent in food industry (Paolucci, Fabbrocini, Volpe, Varricchio, & Coccia, 2012; Paolucci, Fasulo, & Volpe, 2015; Volpe et al., 2012). Fabbrocini et al., (2015, 2012) showed that agar has promising characteristics as a binding agent in the formulation of feeds for the sea urchin *P. lividus*. For this reason, it was used the amount of agar (3%), recommended by those authors (Fabbrocini et al., 2012) to produce diet B in the present work (the jellified mix of macroalgae and vegetables). The bio-components of agar make the pellets to absorb a minimal amount of water and release few residues into the rearing systems, depending on the percentage of agar that is used (Paolucci et al., 2015). Additionally, it is well-accepted by sea urchins. Furthermore, the most interesting feature of agar is that it slows down the progression of the gametogenic cycle and facilitates the synchronization of the gonads reproductive stage (Fabbrocini et al., 2015, 2012). In this study, sea urchins fed with the agar-based diet (diet B) were mostly found in the recovery stage (I), meeting the results of previous studies (Fabbrocini et al., 2015, 2012). The results obtained with the diet B are part of the key points for the success of aquaculture of *P. lividus*, namely as to produce good-quality roe and prevent sea urchins from spawning during transportation to distributors, sellers and consumers. Nevertheless, this subject still needs to be further investigated.

Considering that diet C (maize and spinach) promoted the highest GI values in this and others studies, it may be assumed that an agar-based diet (Fabbrocini et al., 2012) with maize (Basuyaux & Blin, 1998; Silva, 2012), spinach (Sartori & Gaion, 2016), macroalgae (Sartori & Gaion, 2016; Silva, 2012) and vegetables (Prato, Fanelli, et al., 2018) could represent a new promising high-quality and low-cost commercial feed. Although maize presents low levels of protein, it is rich in polysaccharides, which combined with a high protein ingredient can be easily absorbed. Basuyaux and Blin (1998) reported that soluble proteins are as easily absorbed as soluble polysaccharides, thus justifying the fact that they obtained better results by combining maize and seaweeds together than with each one separately. Although proteins have not been quantified in this study, they should also be taken into account as they contribute to the volume growth of the gonads.

According to Boudouresque and Verlaquez (2001), the daily consumption rate of *P. lividus* depends on the availability and type of food. Hence, consumption rates should be higher when the feed is

TABLE 3 Fatty acid composition (%) present in the gonads of *P. lividus* sea urchins, fed with three different diets (diet A—*Codium tomentosum*; diet B—Jellified mix of macroalgae and vegetables; diet C—Maize and spinach) during the experimental trial: saturated fatty acids (SFAs); monounsaturated fatty acids (MUFAs); omega-6 polyunsaturated fatty acids (ω6); omega-3 polyunsaturated fatty acids (ω3).

Fatty acids	Diet A	Diet B	Diet C	Statistical test	*p*-value
SFA					
Myristic (C14:0)	7 ± 1.6	6.3 ± 2	5.5 ± 1.2	$F_{(2,15)} = 1.141$	0.346
Palmitic (C16:0)	25.9 ± 5.5	22.8 ± 4	22.6 ± 1.4	$F_{(2,15)} = 1.091$	0.361
Stearic (C18:0)	19.7 ± 8.8	19.9 ± 11.1	11.4 ± 3.1	$F_{(2,15)} = 1.692$	0.217
Arachidic (C20:0)	1.2 ± 0.3^{a}	1.1 ± 0.3^{ab}	0.7 ± 0.3^{b}	$F_{(2,15)} = 7.728$	0.026
MUFA					
Palmitoleic (C16:1n-7)	1.2 ± 0.8	0.9 ± 0.6	2 ± 0.6	$F_{(2,15)} = 2.709$	0.776
Cis-vaccenic (C18:1n-7)	3.2 ± 1.1^{a}	3.6 ± 1^{a}	11.4 ± 1.3^{b}	$F_{(2,15)} = 0.258$	0.776
Oleic (C18:1n-9)	2.2 ± 1.3	2 ± 0.9	1.7 ± 0.5	$F_{(2,15)} = 85.893$	<0.001
Eicosenoic (C20:1n-9)	7.4 ± 3.8	4.6 ± 1.5	5.6 ± 0.7	$F_{(2,15)} = 1.711$	0.214
Erucic (C22:1n-9)	4.2 ± 1.4	7.8 ± 6.3	2 ± 0.6	$F_{(2,15)} = 3.063$	0.077
ω-6 PUFA					
Linoleic (C18:2n-6)	2.1 ± 0.8^{a}	4.3 ± 1.8^{a}	21.2 ± 3.7^{b}	$F_{(2,15)} = 95.439$	<0.001
Eicosadienoic (C20:2n-6)	0.6 ± 0.6	1.3 ± 2.5	0.3 ± 0.1	$F_{(2,15)} = 0.576$	0.574
ARA—Arachidonic (C20:4n-6)	2 ± 0.6^{a}	1.4 ± 0.5^{ab}	0.7 ± 0.3^{b}	$F_{(2,15)} = 8.890$	0.003
DPA—Docosapentaenoic (C22:5n-6)	–	–	–	–	–
ω-3 PUFA					
α-Linolenic (C18:3n-3)	2.5 ± 1.3	1.9 ± 1.1	2.8 ± 0.7	$F_{(2,15)} = 1.357$	0.393
Octadecatetraenoic (C18:4n-3)	2.7 ± 2.3	4.6 ± 2.8	1 ± 0.5	$F_{(2,15)} = 3.483$	0.057
Eicosatrienoic (C20:3n-3)	7.6 ± 4.4	9.7 ± 3	7.4 ± 1.1	$F_{(2,15)} = 0.833$	0.454
EPA—Eicosapentaenoic (C20:5n-3)	9.8 ± 5.6	7.4 ± 6.5	3.6 ± 2.5	$F_{(2,15)} = 1.872$	0.188
DHA—Docosahexaenoic (C22:6n-3)	0.5 ± 1	0.5 ± 0.5	0.1 ± 0.1	$F_{(2,15)} = 0.818$	0.460
Σ SFA	53.9 ± 13.6	50.1 ± 14.3	40.2 ± 3.3	$F_{(2,15)} = 2.255$	0.139
Σ MUFA	18.2 ± 5.3	18.9 ± 7.1	22.6 ± 0.8	$F_{(2,15)} = 1.267$	0.310
Σ PUFA	27.9 ± 11.6	31.0 ± 10.2	37.1 ± 3.0	$H_{(2)} = 1.450$	0.240
Σ ω3	23.2 ± 11.5	24.1 ± 10.5	14.9 ± 3.9	$F_{(2,15)} = 1.809$	0.198
Σ ω6	4.7 ± 1.3^{a}	6.9 ± 4.7^{a}	22.2 ± 3.7^{b}	$F_{(2,15)} = 1.571$	<0.001
ω3/ω6	5.2 ± 2.9^{a}	4.9 ± 4.2^{b}	0.7 ± 0.3^{b}	$F_{(2,6)} = 4.347$	0.032

Note: Results are presented as mean ± *SD* ($n = 6$). Values signalled by different letters indicate significant differences between diets ($p < 0.05$), for which *F* stands for the results of one-way ANOVA and *H* for the Kruskal–Wallis tests

nutritionally poor and should be lower when it the feed is nutritionally rich (Fernandez & Boudouresque, 2000; Frantzis & Grémare, 1992; Haya & Régis, 1995). The biochemical parameters of the gonads are particularly influenced by the actual stage of gametogenesis, in part because gonads not only are a reproductive organ where gametes are produced, but also store energy-rich reserves (Hughes, Kelly, Barnes, Catarino, & Black, 2006; Russell, 1998; Siliani et al., 2016). However, the fatty acid profile of the gonads at each gametogenic stage is not currently known. Arafa, Chouaibi, Sadok, and El Abed (2012) found that the PUFA content in sea urchins' gonads is significantly affected by temperature, exhibiting high levels during cold months (when *P. lividus* is in the early stages of gametogenesis) and tend to decrease with higher temperatures (when *P. lividus* matures and spawns). In the current work, *P. lividus* specimens fed with diet C (maize and spinach) were mostly found in early stages of gametogenesis (I and III) and presented the highest levels of PUFA in their gonads. This may prove that, in captivity, the gametogenic development of *P. lividus* can be manipulated through feed (Fabbrocini et al., 2015, 2012; Sartori & Gaion, 2016). Notwithstanding, diet A (macroalgae *C. tomentosum*) provided higher levels of EPA (an omega-3 PUFA) in *P. lividus* gonads, which has been proved to be important for several physiological processes (Swanson, Block, & Mousa, 2012). Conversely, diet A itself (macroalgae *C. tomentosum*) presented no EPA in its composition. Therefore, the results obtained suggest that diet A provides an indirect source for this fatty acid to be produced and stored by *P. lividus* in its gonads. Arafa et al., (2012) and Mai, Mercer, and Donlon (1996) demonstrated the importance of macroalgae as a source of fatty acids during the gonadal growth

and gametogenesis. Most marine vertebrates cannot synthesize PUFA, but they may have the ability to further elongate and desaturase dietary PUFA (Sargent, Tocher, & Bell, 2002). PUFA were the major components in the gonads of *P. lividus*, such as α-linolenic acid (C18:3n3), stearidonic acid (C18:4n3), arachidonic acid (C20:4n6) and EPA (C20:5n3). In other similar works, PUFA also have been reported as the major contributors in sea urchin gonads, even though they were not detected or present in low amount in the experimental diets (Prato, Fanelli, et al., 2018). According to some studies (Carboni et al., 2013; Cook et al., 2000; Prato, Fanelli, et al., 2018; Tocher, 2003), sea urchins are able to convert short-chain PUFA (SC-PUFA) into long-chain PUFA (LC-PUFA), such as α-linolenic (C18: 3n3) into stearidonic acid (C18: 4n3), through the Δ6-desaturase. Subsequently, the stearidonic acid (C18: 4n3) undergoes an elongation process, being converted into eicosatetraenoic acid (C20:4n3), which Δ5-desaturase synthesizes into EPA (20:5n-3). Carboni et al. (2013) reported that the input of α-linolenic acid (C18:3n3), through the feed, resulted in higher amounts of EPA in the gonads of *P. lividus*. In the present study, it was possible to verify the same pattern, but with stearidonic acid (C18:4n3). Diet C (maize and spinach) was the richest in α-linolenic acid (C18:3n3), but that was not expressed in the amount of EPA found in the gonads of sea urchin fed with it, which showed similar EPA values to the other experimental animals. This outcome was similar to that obtained by Prato, Fanelli, et al. (2018) in their assay, which assessed the effects of prepared diets and fresh *Ulva* sp. on the gonad yield, nutritional traits and overall quality of *P. lividus*. These authors had an important increase in linoleic acid (C18:2n6c) and α-linolenic acid (C18:3n3) in sea urchins' gonads, but at the same time a decrease in EPA (C20:5n3) and DHA (C22:6n3). In the present situation, diet A (*C. tomentosum*) contained more stearidonic acid (C18:4n3) than the other diets. Being that the case, individuals fed with diet C (maize and spinach) had to expend more energy to synthesize EPA (C20:5n3) from α-linolenic acid (C18:3n3) than those from diet A (*C. tomentosum*), which would only have to synthesize stearidonic acid (C18:4n3). In terms of DHA (C22:6n3) content, all diets promoted lower values in sea urchins' gonads, ranging from 0.1% to 0.5%. It is possible to convert α-linolenic acid (C18:3n3) in DHA (C22:6n3), by elongase and desaturase enzymes, but only in small amounts (Neff et al., 2011).

The human body cannot efficiently produce some omega-3 fatty acids from food sources. It is necessary to obtain adequate amounts through exogenous food sources, like fish and fish oil products (Swanson et al., 2012). Apart from dietary supplements, seafood products remain the major source of n-3 LC-PUFA, since the conversion of α-linolenic acid (C18:3n3) into these long-chain derivatives is low in humans. It has been shown that less than 1% of the α-linolenic acid (C18:3n3) is converted into DHA (C22:6n3) (Burdge & Calder, 2005; Neff et al., 2011; Pawlosky, Hibbeln, Novotny, & Salem, 2001). Studies have shown that EPA and DHA are important for proper embryonic development, including neuronal, retinal and immune function (Swanson et al., 2012). Diets based on high values of ω3/ω6 PUFA ratio are beneficial to animal health (Prato, Chiantore, et al., 2018; Simopoulos, 2006). Different values are recommended for healthy diets, according to distinctive authors, purposes and organisms, but it is always higher than one (Prato, Chiantore, et al., 2018; Simopoulos, 2006). The best ω3/ω6 ratio (2.84) was obtained by Prato, Chiantore, et al. (2018) in sea urchins fed with a formulated diet consisting in krill (30%), *Chaetomorpha linum* (O.F.Müller) Kützing 1845 (15%), starch (50%), vitamin supplement (1%), mineral supplement (3%) and celite (1%). In this study, ω3/ω6 ratio was significantly higher in sea urchins fed diet A (*C. tomentosum* 5.2 ± 2.9), which was also the diet with higher values of this ratio. *P. lividus* fed with diet B (jellified mix of macroalgae and vegetables) also presented high values of ω3/ω6 ratio, despite the diet itself had a ratio lower than 1. Even though diet C (maize and spinach) contained the highest content of PUFA, the diet and the gonads of *P. lividus* specimens fed with it presented the lowest ω3/ω6 ratios.

Despite all the benefits that are associated with the use of high levels of omega-6 PUFA in the formulation of diets for sea urchins and other organisms, some caution and further research should be taken into account. A study has recently been published on the detrimental consequences that this enrichment can bring to the reproductive performance of invertebrates. White, Dworjanyn, Nichols, Mos, and Dempster (2016) studied the effects of n-6 PUFA diet enrichment on the sea urchin *Heliocidaris erythrogramma* (Valenciennes, 1846) and found that females produced smaller oocytes, the larvae presented low survival rates, whereas males' sperm was not viable.

In terms of a marketable product, the best gonad yields are obtained when the nutritive phagocytes of the gonad have attained their greatest degree of mass increase, which occurs just before the gametogenic differentiation (Marsh, Powell, & Watts, 2013). By then, the nutritive phagocytes have accumulated the biochemical components that will be supplied to the developing gametes. Hence, the gonadal tissues will contain high levels of protein, carbohydrate and lipid reserves (Marsh et al., 2013). The development of the nutritive phagocytes is dependent on the assimilation of nutrients derived from the animal diet (Marsh et al., 2013). Thus, an understanding of the biochemical requirements for growth and development of nutritive phagocytes is necessary to optimize the feeds for sea urchins in aquaculture (Marsh et al., 2013). Previous studies have evaluated the variation of lipid levels in sea urchins over time, but the results were contradictory (Martinez-Pita et al., 2010). However, large amounts of spermatozoa and oocytes accumulate in the gonads as they mature, which confers a peculiar lipid composition to the gonadal tissue (Siliani et al., 2016). Martinez-Pita et al., (2010) suggested that the high lipid levels observed during the gonadal growth stages, plus their subsequent decrease during maturation, may indicate that lipids are also used as an energy source. Accordingly, sea urchins fed with diet C (maize and spinach) were mostly in the initial reproductive stages (I and III) of gametogenesis and also showed higher lipid levels. The particularly high standard deviation in the lipid content of the gonads from sea urchins fed with diet C (maize and spinach) resulted mostly from the presence of individuals in recovery and premature stages. In the premature stage, the gonads reach their maximum level of nutrient reserves to initiate the formation of the gametes (Hughes et al., 2006; Siliani et al., 2016).

Of all the diets used in the present assay, diet C (maize and spinach) had an interesting quantity of lipids, fatty acid composition and it was the least consumed by *P. lividus*. This observation confirms that it is possible to obtain successful results with small amounts of feed, further enhancing the sustainability and feasibility of rearing sea urchins in aquaculture. However, diet B (jellified algae and vegetable mix) promoted best results in terms of omega-3 PUFA levels (24.1 ± 10.5%) in the sea urchins' gonads.

In sum, sea urchins fed with diet A (*C. tomentosum*) were mostly in the growth and maturation stages, showed the lowest lipid content, but contained high levels of SFA and EPA. Sea urchins fed with diet B (a jellified mix of macroalgae) were in the reabsorption stage and had the highest values of omega-3 PUFA. At last, sea urchins fed with diet C (maize and spinach) were in the early stages of gametogenesis, had the highest values of lipid content, plus omega-6 PUFAs.

5 | CONCLUSION

In this study, it was found that the use of new food sources for sea urchins appeared to be quite promising, biologically and economically. Both of the laboratory-formulated diet (Diet B) and the use of vegetables (Diet C) were found to be well-accepted by the organisms and no mortality was recorded during the experiment. All the diets used in this study promoted variations in the lipid and fatty acids composition of the sea urchins' gonads. This suggests that sea urchins were able to assimilate nutrients from these innovative diets. The results obtained in this and other studies, substantiate that the introduction of vegetables may be an ideal alternative to the use of cropped macroalgae or conventional feeds. They also demonstrated that a higher nutritional feed may rent more, as it is usually associated with low consumption rates on the behalf of the sea urchins. As to be true, the feeds need to be environmental sustainable, easy to obtain and affordable to be produced. In this study, it was also demonstrated the importance of fatty acids derived from macroalgae, given that sea urchins can convert some short-chain PUFA into long-chain PUFA. To achieve an optimal feed, it is necessary to understand the specific nutritional needs for the different gametogenic stages of *P. lividus*, in order to manipulate them in captivity, according to the desired goal. In this way, it is possible to obtain mature individuals for reproduction and/or repopulation purposes, but also individuals in the initial stages of gametogenesis, which are more adequate for market acceptance of the roe (gonads). Research programs addressing nutrition, physical characteristics of the feed and feed management will be essential for the success of sea urchin aquaculture, as suggested by Lawrence and Lawrence (2004) and McBride (2005). Future studies should include the evaluation of the use of thickeners/binders in the formulation of diets, as well as the evaluation of the sensorial characteristics of the gonads (colour, taste and texture) and consequently their acceptability by the consumer.

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CONFLICT OF INTEREST

None.

DATA AVAILABILITY STATEMENT

The authors declare that the data supporting the findings of this study are available within the article.

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